The functional role of sodium taurocholate co-transporting polypeptide NTCP in the life cycle of hepatitis B, C and D viruses

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Acknowledgments / Funding

This work was supported by Inserm, the University of Strasbourg, the European Union (ERC-2014-AdG-671231-HEPCIR, Infect-ERA hepBccc, EU H2020 Hep-CAR 667273), ANRS (2015/1099), the French Cancer Agency (ARC IHU201301187) and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number R03AI131066. CCC acknowledges fellowships from the Canadian Institutes of Health Research (201411MFE-338606-245517) and the Canadian Network on Hepatitis C. ERV is the recipient of an ANRS fellowship (ECTZ50121).

Abstract

Chronic hepatitis B, C and D virus (HBV, HCV and HDV) infections are a major cause of liver disease and cancer worldwide. Despite employing distinct replication strategies, the three viruses are exclusively hepatotropic and therefore depend on hepatocyte-specific host factors. The sodium taurocholate co-transporting polypeptide (NTCP), a transmembrane protein highly expressed in human hepatocytes that mediates the transport of bile acids, plays a key role in HBV and HDV entry into hepatocytes. Recently, NTCP has been shown to modulate HCV infection of hepatocytes by regulating innate antiviral immune responses in the liver. Here we review the current knowledge of the functional role and the molecular and cellular biology of NTCP in the life cycle of the three major hepatotropic viruses, highlight the impact of NTCP as an antiviral target and discuss future avenues of research.

Keywords: Liver cell biology, bile acid transport, host factor, anti-viral therapy, hepatocytes.

Introduction

Every year, viral hepatitis is estimated to cause around 1.3 million deaths worldwide, mainly through chronic liver disease and hepatocellular carcinoma (HCC). Approximately 95% of these deaths are caused by hepatitis B and C viruses (HBV, HCV) [1]. Despite the availability of an effective vaccine for HBV, 250 million people are chronically infected by the virus worldwide [2]. An estimated 5% of HBV patients are co-infected with hepatitis D virus (HDV), a satellite virus hijacking HBV envelope proteins to assemble its infectious viral particles. HDV co-infection worsens the outcome of HBV infection and treatment of HBV-HDV co-infected patients is less effective [3, 4]. Moreover, around 70 million people are living with chronic HCV infection and, despite the existence of effective curative strategies, the incidence of HCV is still increasing [3].

Remarkable progress has recently been made for treatment of HCV infection. The development and approval of direct acting antivirals (DAAs) specifically targeting viral proteins now allows for HCV cure, but these therapies remain inaccessible for the majority of HCV patients [5]. For chronic HBV infection, two therapeutic approaches are used to suppress viral replication: pegylated interferon and nucleos(t)ide analogues (NUCs). While these treatments allow control of HBV infection, viral eradication is rare and, in most cases, lifelong therapy is required [6]. For patients with chronic HBV/HDV co-infection, the current treatment options are limited to interferon-alpha (IFNα) and its pegylated derivative. Furthermore, although current antivirals decrease the risk of HCC, they are not sufficient to eliminate the risk [7, 8]. In order to effectively combat these hepatotropic viruses,

it is necessary to improve existing therapies and uncover new strategies for prevention and treatment of viral hepatitis.

Alternative strategies against chronic HBV and HCV infection include host-targeting agents (HTA), which target host factors required for viral replication. HTAs have been shown to be promising candidates for the prevention and treatment of infections by various pathogens, including HBV and HCV [9–11]. This approach requires a profound understanding of the viral life cycle and the virus-host interactions involved. Indeed, the identification of the human sodium taurocholate co-transporting polypeptide (NTCP) as a functional receptor for HBV/HDV infection [12, 13] opened perspectives for new antiviral strategies. Several entry inhibitors for treatment of HBV infection targeting NTCP are now in development [14–19]. Furthermore, this crucial discovery has allowed the development of novel infectious model systems that will enable an improved understanding of the complete HBV/HDV viral life cycle [20]. However, the regulatory role of NTCP in HCV host cell infection, and its potential immunomodulatory activities in hepatocytes, should not be overlooked. The aim of this review is to summarize what is known about the interactions of NTCP with three major hepatitis viruses during infection, to describe the molecular mechanisms, and to highlight possible applications in research and therapy.

Sodium taurocholate co-transporting polypeptide, a bile acid transporter

The circulation of bile and bile components between human intestine enterocytes and liver parenchymal cells is known as the enterohepatic circulation (EHC) [21]. In the liver, bile acids are mainly involved in cholesterol metabolism and elimination of toxic compounds [22]. Interestingly, bile acids have also been shown to inhibit interferon (IFN) signaling pathways, resulting in reduced expression of IFN-stimulated genes (ISG) [23, 24]. In hepatocytes, bile acid homeostasis is maintained by the interplay between uptake, synthesis and secretion of bile acids. The major hepatic uptake transporter for conjugated bile acids in humans is sodium taurocholate co-transporting polypeptide (NTCP) [25]. NTCP is predominantly expressed at the hepatic basolateral membrane and is involved in the recycling of bile acids from portal blood to hepatocytes in a sodium-dependent manner [21]. NTCP is a member of the solute carrier family SLC10 and is encoded by *SLC10A1* [26, 27]. *SLC10A1* mRNA is translated into a 349 amino acid glycosylated phosphoprotein with seven or nine transmembrane domains [21, 28–31]. While the exact function of some SLC10 family members remains unknown, all of them are thought to be sodium-dependent transporters [21]. Interestingly, bile acid transport through NTCP can be blocked by small molecules already in clinical use, such as cyclosporine A (CsA, an immunosuppressive drug used in transplantation) or ezetimibe (used for hypercholesterolemia) [16, 32].

Hepatic bile acid metabolism is tightly regulated, including at the transcriptional level (see Figure 1) [33]. Upon bile acid activation, the nuclear factor Farnesoid X Receptor (FXR) indirectly downregulates several target genes through transcriptional induction of the small heterodimer partner (SHP) [34, 35], including the first and rate-limiting enzyme in bile acid biosynthesis cholesterol 7α-hydroxylase (CYP7A1) [36, 37]. FXR also directly activates the expression of the bile salt export pump (BSEP, ABCB11), which is expressed at the apical membrane and secretes conjugated bile acids into the bile canaliculus in an ATP-dependent manner [38, 39]. FXR does not directly interact with the promoter of human *SLC10A1* but induces the expression of different factors to indirectly repress *slc10a1* expression in rat and mouse, although mechanisms of transcriptional regulation of human NTCP remain unknown [40–42]. In hepatic inflammation, the cytokines tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6 downregulate mRNA levels of *SLC10A1* and reduce the transporter protein expression [43–45]. The downregulation of NTCP expression in the human liver has been implicated in several cholestasis pathologies. The reduction of NTCP expression could explain impaired hepatic bile acid uptake, resulting in cholestatis and jaundice. Several studies have shown a downregulation of bile salt transporters in primary biliary cirrhosis [46, 47]. Interestingly, a recent study showed a suppression of NTCP expression via cyclin D1 in hepatocellular carcinoma (HCC) [48]. These data may explain the low expression level of NTCP in HCC-derived cell lines, such as Huh7 and clones or HepG2.

The localization and membrane expression of NTCP is controlled by post-translational mechanisms [49]. For example, cyclic adenosine monophosphate (cAMP) plays a role in stimulating the dephosphorylation and membrane translocation of NTCP (see Figure 1) [50–52]. Sequencing analysis of NTCP revealed the existence of several ethnic-dependent single nucleotide polymorphisms (SNPs) which may alter NTCP activities [53]. For example, mutation S267F, found in 7.5% of allele frequencies in Chinese Americans, is associated with an almost complete loss of bile acid uptake function. However, no pathologies have been described resulting from these NTCP polymorphisms and their clinical roles remain controversial [54]. Besides its major role in the bile acid uptake system, Yan *et al.* described the crucial role of NTCP on HBV and HDV entry [12]. For the time being, NTCP remains the only described HBV and HDV entry receptor.

NTCP is a host factor for HBV/HDV infection

Hepatitis B virus is the prototypic member of the *Hepadnaviridae* family of small enveloped hepatotropic DNA viruses. Its envelope consists of three different forms of the HBV surface protein (HBsAg) – the small (S), middle (M) and large (L) proteins. Importantly, the preS1-domain of L envelope protein is known to bind the hepatocyte cell surface and is required for HBV and HDV entry [55]. The HBV capsid is comprised of HBV core protein (HBcAg) and encapsidates a partially double-stranded relaxed circular DNA (rcDNA) genome of 3.2 kilobases. Upon infection of hepatocytes, genomic rcDNA is converted into covalently closed circular DNA (cccDNA), a minichromosome-like structure that persists in the nucleus as a central transcription template for all viral RNAs [56]. The presence of cccDNA in the nucleus is thought to be responsible for viral rebound after withdrawal of NUC therapy that targets reverse transcription, a late step in the HBV life cycle. Therefore, removal of cccDNA from HBV-infected hepatocytes will be essential to achieve the goal of HBV cure [57].

HDV is a defective hepatotropic virus which depends on HBV surface proteins for assembly of infectious virions and viral entry [58]. The HDV genome is a negative single-stranded circular RNA of nearly 1700 nucleotides containing one functional open reading frame, which encodes the hepatitis delta protein (HDAg) expressed in small and large form. Replication of HDV RNA and transcription of HDAg mRNA in the nucleus depends on host cell polymerases, including DNA-dependent RNA polymerase II. Both forms of the delta protein are then produced and reimported in the nucleus where they bind to genomic RNA to form the ribonucleoprotein (RNP), which is then exported into the cytoplasm and is associated with HBV envelope proteins to form a mature HDV virion [59]. Thus, HDV enters hepatocytes using the same pathways as HBV, and depends on the same host factors for host cell binding and entry. HDV is therefore a useful surrogate model for HBV entry.

The first step of viral infection is virion binding to attachment factors and receptors at the host cell surface. This specific interaction between viral surface proteins and host entry receptors often determines the tissue tropism and host range of the virus [60]. HBV and its satellite virus HDV share HBV envelope proteins and are known to exclusively infect human, chimpanzee and tree shrew (*Tupaia belangerii*) hepatocytes, suggesting the involvement of species- and liver-specific cell surface factors in the common entry process of these viruses [20]. Two elements of the HBV envelope proteins are necessary for interaction with these factors. One determinant of infectivity resides in the surface-exposed cysteine-rich antigenic loop (AGL), a polypeptide located in the S domain of all three envelope proteins [61, 62]. The second known infectivity determinant is a receptor binding site in the N-terminal pre-S1 domain of the L-HBsAg [55]. This domain is post-translationally modified by addition of myristic

acid [63], and this myristoylation is essential for virion infectivity [64, 65]. A synthetic myristoylated peptide comprising the N-terminal amino acids 2 to 78 of the pre-S1 domain prevents HBV infection [66].

As for many viruses [67, 68], HBV/HDV infection requires the initial attachment to the glycosaminoglycan (GAG) side chains of heparan sulfate proteoglycans (HSPGs) [69]. Both the antigenic loop of all HBV envelope proteins and the preS1-region of HBsAg-L are involved in this interaction [69, 70]. Indeed, glypican-5 (GPC5), a member of the glypican family of HSPGs, acts as an entry factor for HBV and HDV (see Figure 2) [71]. After this initial step of HBV/HDV attachment to HSPGs, the virions bind to a high-affinity receptor via the preS1-domain [72], allowing uptake into hepatocytes. Despite the discovery of several preS1-interacting proteins that did not affect HBV infectivity [73–78], the identity of the HBV/HDV entry receptor remained unclear until 2012, when Yan *et al.* identified NTCP as a functional receptor for HBV and HDV infection. Using a labeled preS1 peptide as a bait in *Tupaia* hepatocytes, a mass spectrometry purification of preS1-bound proteins, and validation in human hepatocytes, it was shown that NTCP specifically interacts with the HBV receptor-binding domain preS1, allowing viral entry [12]. Zhong *et al.* showed that *Tupaia* NTCP mediates entry of woolly monkey HBV, indicating that NTCP orthologs act as a common cellular receptor for known primate hepadnaviruses [79]. Differential gene expression patterns between non-susceptible undifferentiated and susceptible differentiated HepaRG cells validated the role of NTCP as a specific receptor for HBV and HDV [13]. Moreover, silencing of NTCP in primary *Tupaia* hepatocytes (PTH) or differentiated HepaRG cells inhibits HBV and HDV infection [12, 13]. Exogenous expression of NTCP directly renders non-susceptible hepatoma cell lines susceptible to HBV and HDV infection, while entry inhibitors derived from the preS1 peptide efficiently inhibit this infection [12]. In addition, the S267F mutant of NTCP, conferring a loss of bile acid uptake function, is significantly associated with resistance to chronic hepatitis B and decreased risk of cirrhosis and liver cancer development, supporting the role of NTCP as cellular receptor for HBV in human infection [80–82]. However, S267F homozygote patients can still be infected by HBV, suggesting the existence of alternative receptors allowing viral entry in the absence of functional NTCP [83].

Interestingly, expression of human (but not mouse) NTCP in non-susceptible hepatocarcinoma cells confers limited susceptibility to infection. For robust infection, addition of dimethyl sulfoxide (DMSO) to culture medium is essential [13]. The fact that human hepatoma cell lines HepG2 and Huh7 are not susceptible to HBV and HDV infection without exogenous expression of NTCP is consistent with reports that NTCP expression is reduced in human hepatocellular carcinoma cells [48, 84]. NTCP expression rapidly decreases over time following isolation of cultured PTHs, which supports observations that primary human hepatocytes (PHH) remain

susceptible to HBV infection *in vitro* only for a few days after isolation [12, 85]. Considering the predominant expression of NTCP in the liver, this receptor is likely to contribute to the hepatotropism of both viruses [12]. In addition, NTCP protein sequences vary among mammalian species, which might contribute to the narrow species tropism of HBV and HDV infection. For example, monkey NTCP does not support HBV and HDV infection despite a high protein sequence homology to human NTCP. Replacing amino acids 157–165 of nonfunctional monkey NTCP with the human counterpart conferred susceptibility to both HDV and HBV infection [12]. The fact that hepatocytes from cynomolgus and rhesus macaques and pigs become fully susceptible to HBV upon hNTCP expression indicates that NTCP is the key host factor limiting HBV infection in these species [86].

As a key host factor enabling HBV and HDV infection *in vitro*, the discovery of NTCP has been crucial for the development of novel animal models supporting virus infection. Indeed, only chimpanzees and *Tupaia* can experimentally support HBV and HDV infections [87]. The state-of-the-art mouse model for the study of HBV/HDV consists of liver-engrafted humanized chimeric uPa/SCID or FRG mice, which support virus entry and replication, but lack an efficient immune system, limiting the study of virus-host interactions [87]. The recent development of human NTCP-expressing transgenic mice opened perspectives for the development of novel immune-competent animal models for the investigation of HDV infection and HDV-induced pathogenesis *in vivo* [88]. As HBV infection is limited in mouse cells expressing hNTCP, probably due to the lack of a key host factor [89], it should be noted that hNTCP-transgenic mice are not susceptible to HBV infection. Recently, an elegant study demonstrated that vector-mediated expression of hNTCP in the hepatocytes of rhesus macaques conferred susceptibility to HBV infection, providing a robust and relevant model for the study of HBV infection, including its interaction with adaptive immunity and the understanding of viral clearance [90].

Overall, NTCP was identified as the long-sought preS1-specific HBV receptor contributing to HBV liver tropism and species specificity [13]. Targeting the interactions between the HBV preS1-domain and its receptor NTCP required for HBV/HDV entry is a promising strategy to block viral entry for both viruses.

NTCP as a therapeutic target for HBV/HDV infection

Even before the identification of NTCP as HBV/HDV receptor, entry inhibitors derived from the HBV preS1 were shown to efficiently inhibit HBV infection *in vitro* and *in vivo* [91, 92]. One of these compounds, the myristoylated preS1-derived peptide (also called Myrcludex B or MyrB), efficiently prevents HBV dissemination *in vivo* and hinders amplification of the cccDNA pool in infected human hepatocytes [14]. MyrB is the first HBV/HDV entry inhibitor targeting NTCP to reach clinical trials [93], where it was shown to have a good safety profile with a mild

and reversible elevation of serum bile acid salts [93, 94]. Phase IIa clinical studies revealed a marked antiviral effect of MyrB, as measured by HDV RNA, HBV DNA and improvement of biochemical disease activity (ALT), when used in combination with IFN therapy, although there was no significant decrease in HBsAg levels. In monotherapy, however, MyrB did not show significant antiviral activity [94]. Further studies are necessary to confirm these results obtained in small patient cohorts [95].

Importantly, the identification of NTCP as the first HBV/HDV entry receptor has accelerated the discovery and development of several new potential entry inhibitors. Binding of myristoylated preS1-derived peptide to NTCP was shown to interfere with the physiological bile acid transport function of NTCP, indicating that NTCP-inhibiting drugs might be able to block HBV infection [96]. In a study evaluating FDA approved therapeutics with documented inhibitory effect on NTCP cellular function against HDV entry, three of these molecules (irbesartan, ezetimibe, and ritonavir) inhibited HDV infection *in vitro* [97]. The inhibitory effect of ezetimibe on HBV infection had already been described previously without understanding its interactions with NTCP [98]. In 2014, Watashi *et al.* evaluated the effect of compounds on the early phase of the HBV life cycle to identify cyclosporine A as an HBV entry inhibitor targeting NTCP [15]. In the same year, Nkongolo *et al.* characterized the effect of cyclosporine A, a cholestasis-inducing drug inhibiting NTCP bile acid transport [32, 97, 98], against HBV/HDV infection and found that inhibition of entry resulted from interference with the NTCP receptor [16]. The screening of FDA/EMA-approved drugs or small molecules for interaction with NTCP allowed the identification of several additional potential HBV/HDV entry inhibitors targeting NTCP [18, 19]. All of these NTCP-targeting HBV/HDV entry inhibitors concomitantly inhibit the transporter function of NTCP and impair bile acid uptake into hepatocytes, increasing the risk of adverse effects. NTCP-deficient mice and a patient with NTCP deficiency were shown to exhibit an elevated level of serum bile acids and to develop related pathologies including growth retardation and hypercholanemia [101, 102].

Two different strategies to selectively inhibit HBV entry without impairing bile acid uptake have been suggested recently. Shimura *et al.* showed that cyclosporine A derivatives SCY450 and SCY995 inhibit HBV/HDV entry without interfering with the NTCP transporter activity (see Figure 2) [17]. Tsukuda *et al.* identified an oligomeric flavonoid, proanthocyanidin (PAC) and its analogs, as a new class of entry inhibitors, which directly target the preS1-domain of the HBV large envelope protein and thereby prevent its attachment to NTCP. By directly targeting HBV particles, PAC impairs HBV infectivity without affecting the NTCP-mediated bile acid transport activity [103]. Further studies are required to determine if these novel inhibitory strategies will show efficacy *in vivo* and in clinical studies in co-treatment with NUC therapy.

NTCP is a host factor for HCV infection

Hepatitis C virus is an enveloped single-stranded positive-sense RNA virus in the *Flaviviridae* family. The host-cell derived lipid envelope contains the two viral envelope glycoproteins, E1 and E2 [104]. Within the envelope, an icosahedral capsid contains the RNA genome of 9.6 kilobases. Like HBV and HDV, attachment of HCV to hepatocytes is mediated by HPSGs on the host cell surface [105–107]. Following attachment, the envelope glycoprotein E2 mediates interactions with a series of specific cellular entry factors, including CD81 and claudin-1 (see Figure 2) [108–111]. HCV is internalized via endocytosis in a clathrin- and dynamin-dependent process [112]. Following fusion with early endosomal membranes, the HCV genome is released into the cytosol, where it is translated into a polyprotein cleaved by viral and host proteases. The HCV genome is replicated directly into RNA without passing through a DNA intermediate [113]. Therefore, HCV entry and replication steps are very distinct from those described for HBV/HDV. Nonetheless, the mutual hepatotropism of these three viruses mediated by tissue specific factors suggests a possible overlap in usage of common hepatocyte specific host factors like NTCP.

Following establishment of the pivotal role of NTCP for HBV and HDV entry into hepatocytes, a recent study identified a role for NTCP in HCV infection (see Figure 2). Exogenous overexpression or silencing of NTCP increased or decreased HCV infection *in vitro*, respectively [114]. Unlike HBV, however, no direct interaction between HCV envelope proteins and NTCP was identified. Instead, the bile acid transporter function of NTCP was found to be important for HCV entry [114]. Bile acids are known to modulate cellular antiviral responses by inhibiting interferon (IFN) type I signaling and thereby decreasing the expression of IFN-stimulated genes (ISGs) [23, 24]. NTCP was shown to regulate HCV infection by inducing the bile acid-mediated repression of ISG expression in hepatocytes, including IFITM1, IFITM2 and IFITM3 [114]. These transmembrane proteins are known to restrict the entry of several viruses, including HCV [115]. IFITM1 blocks the interaction between HCV and its receptors [116], whereas IFITM2 and IFITM3 inhibit entry at a post-endocytosis step by blocking the release of virions into the cytoplasm [117]. NTCP facilitates HCV infection by modulating innate antiviral responses via its bile acid transport function. As bile acids have been shown to enhance HCV replication [118], it is likely that NTCP expression and activity modulates HCV infection through a multimodal mechanism of action. Interestingly, MyrB-mediated inhibition of NTCP blocks the import of bile acids, which in turn stimulates the expression of ISGs, inhibiting HCV entry and infection [114]. However, it still needs to be determined whether the inhibition of NTCP-mediated bile acid entry affects the HBV life cycle through similar mechanisms as

described for HCV. The potential of NTCP-targeting antivirals to enhance innate antiviral responses and to engage the host immune system to clear infection may be a useful property for the treatment of all hepatotropic viruses, including HBV, HCV and HDV.

Conclusions

The discovery of NTCP as the first HBV/HDV receptor was a milestone in the study of the life cycle of these viruses. This landmark discovery enabled significant progress in understanding HBV/HDV entry and virus-host interactions. Moreover, based on this discovery, novel infectious model systems based on transduced cell lines stably expressing NTCP have been developed which allow detailed study of the early steps of the viral life cycle. By allowing the study of authentic infection in cell lines, these model systems will help to understand the formation and degradation of HBV cccDNA, which is a key target to achieve the ultimate goal of HBV cure. Robust human NTCP-expressing animal model systems will enable the *in vivo* validation of virus-host interactions and antiviral therapies. Moreover, NTCP has been established as an antiviral target, and several molecules targeting NTCP are in clinical development with the goal to improve current therapies in the future. The recent discovery of NTCP as a host-dependency factor in HCV infection underscores its essential role in virus-hepatocyte interactions.

Author contributions

CFE, LH, CCC, ERV, CS, TFB wrote the manuscript.

Conflicts of interest

The authors have no conflicting interests to disclose.

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629 **Figure legends**

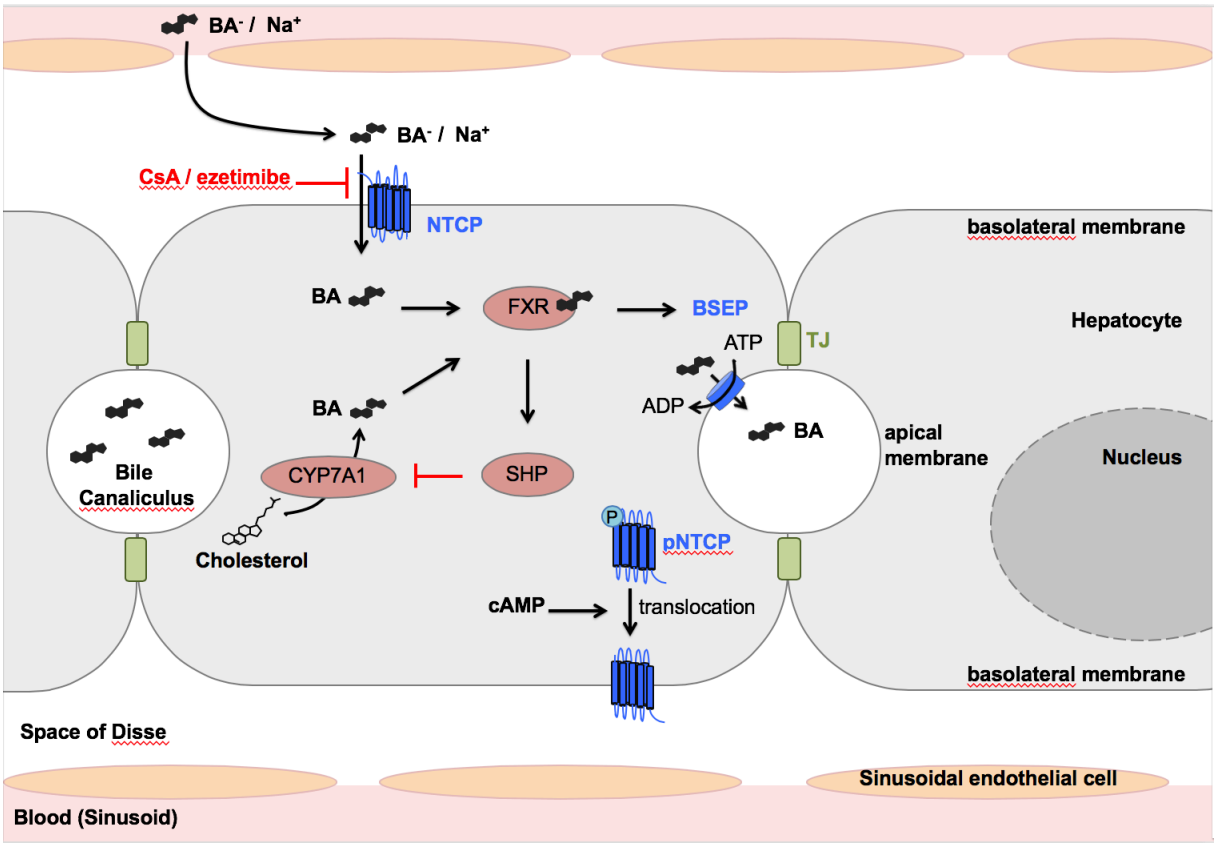
630 **Fig. 1 Model of the functional role of NTCP in hepatic bile acid transport and metabolism**. Transport of bile
631 acids from portal blood into hepatocytes *via* NTCP depends on a sodium gradient and is inhibited by CsA or
632 ezetimibe. Secretion into the bile canaliculus *via* bile salt export pump (BSEP) in an ATP-dependent manner and
633 synthesis from cholesterol are regulated by bile acid-mediated activation of FXR. cAMP mediates
634 dephosphorylation and membrane translocation of NTCP. NTCP: Sodium taurocholate co-transporting
635 polypeptide; BSEP: bile salt export pump; FXR: Farnesoid X Receptor; SHP: small heterodimer partner; CYP7A1:
636 cholesterol 7α-hydroxylase; BA: bile acid; TJ: tight junction; CsA: cyclosporin A; cAMP: cyclic adenosine
637 monophosphate

638

639 **Fig. 2 Model of interactions between NTCP and the entry of HBV, HDV, and HCV in hepatocytes.** After
640 initial attachment to HSPG including GPC5, HBV and HDV virions bind to the receptor NTCP through the preS1-
641 domain of the large envelope protein. NTCP inhibitors CsA and ezetimibe block viral entry like preS1-derived
642 MyrB and CsA-derived SCY995. NTCP modulates HCV infection by interfering with innate immune responses.
643 Bile acids interfere with the IFN signaling pathway and thereby favor HCV entry. Inhibition of NTCP-mediated
644 bile acid import into hepatocytes promotes inhibition of HCV entry through the upregulation of ISGs including
645 *IFITM*s. HBV: hepatitis B virus; HCV: hepatitis C virus; HDV: hepatitis D virus; HSPG: heparan sulfate
646 proteoglycan; GPC5: glypican-5; NTCP: Sodium taurocholate co-transporting polypeptide; MyrB: myrcludex B;
647 CsA: cyclosporin A; SCY995: synthesized CsA derivative 995; IFN: interferon; IFNAR: IFN-α/β receptor; JAK:
648 Janus kinase; STAT: signal transducer and activator of transcription; IRF9: Interferon regulatory factor 9; ISRE:
649 IFN-sensitive response element; ISG: IFN-stimulated gene; IFITM: IFN-induced transmembrane protein; CLDN1:
650 Claudin 1; CD81: cluster of differentiation 81; BA: bile acid; TJ: tight junction

651

652 **Figure 1**



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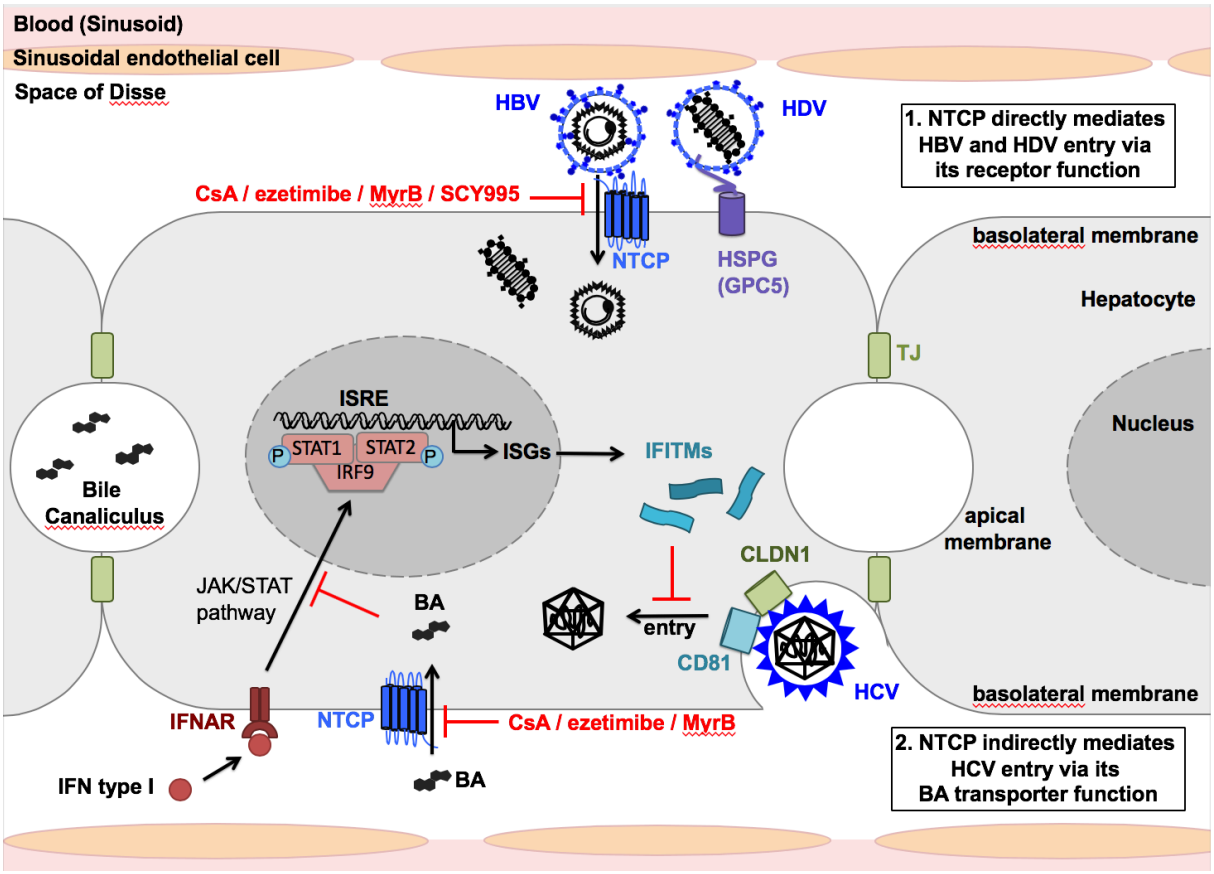
655 **Figure 2**



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